agonistic or antagonistic antibodies which can be used to alter responses to IL-1. "Given that IL-1R AcM is involved in IL-1 signal transduction, antibodies directed against IL-1R AcM are expected to behave as agonists or antagonist[s] of IL-1 activity." Specification at page 44, lines 15-17. This asserted utility was not addressed in the utility rejection as discussed in Paper No. 20.

The use of the claimed polynucleotides to produce antibodies that alter responses to IL-1 constitutes a specific, substantial, and credible utility, and for this reason alone the utility rejection should be withdrawn. For example, antibodies that antagonize IL-1 are expected to reduce the severity of diseases associated with infection, inflammation, and metabolic disturbances. *See* specification at page 43, lines 11-24. Moreover, it is reasonable to expect that antibodies to human soluble IL-1R AcM can antagonize IL-1 action. Antibodies to murine IL-1R AcM, whose sequence is 85% identical and 94% similar to the human IL-1R AcM of the instant invention, were shown to block IL-1ß binding to murine type I IL-1R. Greenfeder et al., J. Biol. Chem. 270:13757-13765 (1995)), cited in specification at page 44, line 19. Conversely, antibodies to IL-1R AcM which agonize IL-1 would be expected *inter alia* to enhance host responses to infection, regulate liver metabolism, regulate insulin secretion by pancreatic ß cells, and enhance wound healing by increasing proliferation of fibroblasts. *See* specification at page 42, line 17 through page 43, line 9. Thus, the production of either agonistic or antagonistic antibodies to the polypeptides encoded by the claimed polynucleotides is a specific, substantial, and credible utility which was asserted in the specification.

A further utility asserted in the specification, but not addressed in the Office Action, is the use in the diagnosis of IL-1 related diseases. The use of polynucleotides and antibodies of the invention for diagnosis and monitoring of IL-1R AcM gene expression is disclosed in the specification, for example, at page 12, lines 15-18; at page 13, lines 16-17; at page 18, lines 13-21; page 45, lines 3-6; and in Example 4. In support of this asserted utility, a publication by Jensen et al. (J. Immunol. 164:5277-5286 (2000), attached as Exhibit A) has demonstrated that, in response to stress, human liver cells increase their expression of soluble IL-1R AcM and decrease their expression of membrane-bound IL-1R AcM. Thus, the asserted utility of diagnosing IL-1 related diseases by determining the expression level of soluble IL-1R AcM is also a specific, substantial, and credible utility.

The first of the asserted utilities discussed in the Office Action is described as "[t]o search for drugs as ligands or antagonists of the polypeptide encoded by the claimed

polynucleotide." Paper No. 20 at page 5. The Examiner has stated that this utility is credible and specific, but not substantial.

> Significant further eperimentation would be required of the skilled artisan to characterize the protein and search for ligands. There is no disclosure for example, of how to assay for ligand binding and possible transduction mechanisms. It is not known all classes of agonists to use or what measurements to perform. Since this asserted utility is not presented in mature form so it could be readily used in a real world sense, the asserted utility is not substantial.

Paper No. 20 at page 5, lines 12-15. Applicants respectfully disagree. All of the materials and methods needed to perform an agonist/antagonist screening assay are described in the specification or were known in the art at the time of filing. For example, assays to characterize the number and affinity of IL-1 binding sites are described in the specification at page 19, lines 2-5; at page 19, lines 24-29; and at page 33, line 6 through page 35, line 20. The choice of IL-1ß as the ligand is specified *inter alia* at page 19, line 24 and throughout the two paragraphs at page 19, line 30 through page 20, line 20. Appropriate recombinant cell lines for the binding assay are described at page 19, lines 6-23 and at page 33, line 18 through page 34, line 15. Candidate agonists are described at page 36, lines 1-5, and include mutants of IL-1ra and IL-1 mutants described in publications prior to the time of filing. Candidate antagonists are described at page 35, lines 23-30, and include IL-1ra, antibodies that bind IL-1R AcM, and antibodies that bind type I IL-1R. Generation of the relevant antibodies is described at page 36, line 28 through page 39, line 10. Thus, it was clear from the disclosure and the knowledge in the art at the time of filing how to carry out screening for agonists and antagonists without the need for further experimentation.

The second of the asserted utilities in the Office Action was "[f]or the production of antibodies." Paper No. 20 at page 5, line 17. The Examiner has stated that this asserted utility is credible and substantial, but not specific because "the specification discloses nothing specific and substantial about the polypeptide" used to generate the antibodies. Paper No. 20 at page 5, lines 18-19. Applicants respectfully disagree. The specification discloses numerous specific properties of the polypeptides encoded by the claimed polynucleotides which distinguish them from other polypeptides. Most notably, the human soluble IL-1R AcM is 85% identical and 94% similar in amino acid sequence to murine IL-1R AcM, which has been shown to enhance the binding affinity of IL-1ß for the type I IL-1 receptor. Antibodies to the murine IL-1R AcM were shown to block the binding of IL-1ß to type I IL-1 receptor. Greenfeder et al., *supra*. Because of the high degree of sequence

identity and similarity, it is reasonable to conclude that the human and murine IL-1R AcM polypeptides share these characteristics, which alone are sufficient to describe the specific use for antibodies to human IL-1R AcM.

The third allegedly asserted utility described in the Office Action is "[t]o produce a variant or chimeric nucleotide or polypeptide." Paper No. 20 at page 6, line 1. The Examiner has stated that this asserted utility is credible but not specific or substantial because "the specification discloses nothing specific or substantial for the variant nucleotide and polypeptide." Applicants disagree. The specification states that variant polypeptides, or polypeptides encoded by variant polynucleotides, "show substantial soluble IL-1R AcM polypeptide activity." Specification at page 26, line 10. Because the human soluble IL-1R AcM polypeptide itself has specific and substantial utility, as discussed above, it is clear that variants which possess "substantial soluble IL-1R AcM polypeptide activity" will possess the same specific and substantial utility.

Finally, the Office Action discusses a fourth allegedly asserted utility, which is "[t]o search for physiological activity of the claimed polynucleotide encoding the polypeptide, or its binding partners." Paper No. 20 at page 6, lines 7-8. Applicants' representatives are not aware of any such assertion of utility in the specification. The specification asserts at least one function of human soluble IL-1R AcM, which is to increase the binding affinity of type I IL-1R for IL-1ß.

For the reasons discussed above, Applicants respectfully request the withdrawal of this rejection.

Rejection of Claims 20-29, 38, 39, 49-58, and 60-73 Under 35 U.S.C. § 112, First Paragraph – Enablement

I. Claims 20-29, 38, 39, 49-58, and 60-73 are rejected under 35 U.S.C. § 112, first paragraph. The Office Action states that because the claimed invention allegedly is not supported by either a specific, substantial and credible asserted utility or a well-established utility, one skilled in the art would not know how to use the claimed invention. Applicants respectfully disagree and traverse the rejection.

For the reasons discussed above in response to the rejection under 35 U.S.C. § 101, Applicants assert that the instant invention does fulfill the utility requirement of 35 U.S.C. § 101. The Examiner "should not impose a 35 U.S.C. § 112, first paragraph, rejection grounded on a 'lack of utility' basis unless a 35 U.S.C. § 101 rejection is proper." M.P.E.P.

§ 2107 (IV) at 2100-28. Therefore, because the claimed invention complies with the utility requirement of 35 U.S.C. § 101, the rejection based on the alleged lack of utility of the claimed invention should be withdrawn.

II. Furthermore, the Examiner alleges that the claimed polynucleotides are not enabled because an algorithm (the "antigenic index") has been used to identify potential antigenic sites on the encoded polypeptides and because homology has been used to predict the function of the encoded polypeptides. Applicants respectfully disagree also with these grounds for the rejection.

The Examiner has stated that "identifying possible epitopes on a polypeptide by use of a computerized algorithm is unreliable as far as correct identification of the epitopes, and does little to describe an enabled utility or function for the polypeptide." Paper No. 20 at page 7, lines 2-5. The Examiner further states that this conclusion is justified by a paper by Daniel et al. (Virology 202:540-549 (1994)), which allegedly concludes that certain features of a polypeptide, such as hydrophobicity, surface exposure, and chain flexibility are poorly predictive of antibody binding sites. However, a closer examination of the Daniel et al. paper reveals that the Examiner's generalizations about the usefulness of such algorithms are not supported. Daniel et al. used a combination of nine algorithms in an effort to identify novel epitopes on the S glycoprotein of murine hepatitis virus. First, only a single protein was tested, so there is no justification for generalizing the findings of Daniel et al. to all proteins. Second, the nine algorithms used by Daniel et al. did not include the Jameson algorithm employed by the instant inventors. Third, of the 15 algorithm-identified epitopes tested by Daniel et al., 12 were in fact "highly immunogenic" (Daniel et al., page 542, right column, last paragraph); in this respect, it should be noted that the immune responses obtained for the 15 synthetic peptides were highly dependent on the carrier protein used. Fourth, the "failure" of the algorithms in the Daniel et al. study was only with respect to the inability of the algorithms to predict epitopes resulting in either neutralizing antibodies or antibodies which could protect mice from challenge with virus. Thus, the results of Daniel et al. cannot be generalized to the prediction of antibody binding sites for all proteins, or to the prediction of epitopes of IL-1R AcM that are capable of generating either agonistic or antagonistic antibodies. Moreover, even in the unlikely event that none of the predicted epitopes described in the specification actually corresponds to an antibody binding site, the instant claims would still be enabled. The use of sythetic peptides to generate antibodies is merely one approach. The antibodies of the invention can also be obtained using the entire

IL-1R AcM polypeptide, or a wide variety of possible fragments thereof, as the immunogen. This approach does not even require the use of predicted epitopes.

The Examiner has presented a number of publications which point out potential pitfalls in the prediction of protein function based on homology. Each of these will be discussed below. However, none of these publications stands for the proposition for which the Examiner cites them, which is: "Generally, the art acknowledges that function cannot be predicted based solely on structural similarity to a protein found in the sequence databases." Paper No. 20 at page 7, lines 11-12. These publications describe certain exceptions to the widely recognized principle that, generally, protein function can be predicted based on homology, particularly where that homology is very high, such as the 85% identity and 94% similarity between human soluble IL-1R AcM of the instant invention and murine IL-1R AcM.

Skolnick et al. (Trends in Biotech 18:34-39 (2000)) is cited for the proposition that "knowing the protein structure by itself is insufficient to annotate a number of functional classes, and is also insufficient for annotating the specific details of protein function (see Box 2, p. 36)." Paper No. 20 at page 7, lines 13-15. However, closer analysis of Box 2 of Skolnick reveals that it addresses the problem of trying to predict protein function based on assignment to one of five protein superfamilies which share certain folding motifs. Each of these five superfamilies contains a large number of protein families, and each family contains a number of proteins. There is no indication that the folding-based superfamilies evaluated by Skolnick have any significant degree of sequence homology. Similarly, Brenner (Trends in Genetics 15:132-133) lacks relevance because it addresses only functional comparisons within superfamilies, not between two proteins with a high degree of sequence homology.

Bork (Genome Res 10:398-400 (2000)) "states that the error rate of functional annotations in the sequence database is considerable, making it even more difficult to infer correct function from a structural comparison of a new sequence with a sequence database." Paper No. 20 at page 7, lines 15-18. This is not relevant to the instant case, where function has not been predicted from a functional annotation in a database, but based on published experimental data for the well characterized mouse IL-1R AcM (*e.g.*, Greenfeder et al., *supra*). Relatedly, Bork et al. (Trends in Genetics 12:425-427 (1996)) also lacks relevance because it merely questions database annotations based on structural similarity of a small domain, not 85% overall sequence identity as in the instant case. Doerks et al. (Trends in

Genetics 14:248-250 (1998)) is cited for the concern that "structural similarity often does not necessarily coincide with functional similarity." Paper No. 20 at page 7, last line, through page 8, line 1. However, Doerks et al. also addresses only the use of functional annotations in databases. This is again not relevant to the instant case, which is not based on a database annotation. Similarly, the Examiner states that Smith et al. (Nature Biotech. 15:1222-1223 (1997)) notes that "there are numerous cases in which proteins having very different functions share structural similarity due to evolution from a common ancestral gene." However, Smith et al. merely considered pitfalls related to automated annotation of large databases; the Examiner's citation from Smith et al. does not state a general rule for all sequence comparisons that can be applied to the instant case.

For the reasons stated above, Applicants submit that the Examiner has failed to meet the requirement for a *prima facie* enablement rejection, because none of the references cited actually stands for the premise for which it has been cited. The conclusions stated for Skolnick et al. refer to functional comparisons within folding motif superfamilies. The conclusions stated for Brenner also relate to superfamilies of proteins. The conclusions stated for Bork, Bork et al., Doerks et al., and Smith et al. are all limited to the uncritical copying of functional annotations in large, computer-generated databases. None of the cited references is relevant to predictions based on a protein which is 85% identical and 94% similar at the amino acid sequence level to a well-characterized protein.

III. The Examiner also alleges that "[t]he specification does not teach the skilled artisan how to use the claimed polypeptide for any purpose." Paper No. 20 at page 8, lines 17-18. The reason is allegedly that no particular disease states are disclosed for which the level or form of the claimed polypeptides is diagnostic, the physiology of knock-in and knock-out animals is not described, and no treatment protocols are provided for the disclosed polypeptides. *See* Paper No. 20 at page 8, lines 18-21. Finally, the Examiner alleges that not a single one of the eight *Wands* factors weighs in favor of enablement of the instant claims. *See* Paper No. 20 at page 9, lines 3-17. Applicants respectfully disagree also with these grounds for the rejection.

Initially, Applicants respectfully remind the Examiner that the instant claims read on polynucleotides, not polypeptides. The claimed polynucleotides encode polypeptides that can be employed to generate or select antibodies that bind IL-1R AcM. As described above, such antibodies can be either agonistic or antagonistic with respect to activities produced by IL-1 upon its binding to type I IL-1 receptors.

The specification discloses a number of specific diseases which can be treated with agonistic or antagonistic antibodies to human soluble IL-1R AcM, including rheumatoid arthritis, osteoarthritis, scleroderma, skin diseases such as psoriasis and epidermal fungal infections, infectious diseases in general, diseases of liver metabolism, and tissue injury (*i.e.*, wound repair). *See* specification at page 42, line 16 through page 43, line 9. Numerous well-characterized effects of IL-1 in animals and humans are summarized at page 43, line 14, through page 44, line 14. Therapeutic applications of antibodies to IL-1R AcM, including relevant pharmaceutical compositions, administration, and dosing, are described at page 44, line 15 through page 47, line 7. Thus, contrary to the Examiner's assertions, the specification teaches how to use the claimed polynucleotides, and the antibodies resulting from them, in the treatment of several specific diseases.

With regard to the *Wands* factors, the Examiner's concern appears to lie chiefly with (1) the level of predictability of establishing biological activity based on structure and (2) the breadth of the claims, which fail to recite a biological activity. The Examiner has failed to provide any specific basis for concluding that the other *Wands* factors weigh against enablement of the instant claims. Applicants believe that the level of predictability of human soluble IL-1R AcM activity is reasonable in light of its great similarity (85% identity and 94% similarity at the amino acid level) to murine IL-1R AcM, whose function as an enhancer of IL-1 binding affinity was well characterized at the time of filing. As to the breadth of the claims, Applicants point out that while the pending claims do not recite a particular biological activity, they are all directed to polynucleotides encoding specified portions of SEQ ID NO:2 or the amino acid sequence encoded by the deposited cDNA. None of the pending claims are directed to polynucleotides encoding variants of either SEQ ID NO:2 or the amino acid sequence encoded by the deposited cDNA. Thus, Applicants believe that evaluation of the *Wands* factors leads to the conclusion that the instant claims are enabled.

For all the reasons discussed above, Applicants believe that the pending claims are fully enabled with respect to the use of the claimed polynucleotides. Accordingly, withdrawal of this rejection is respectfully requested.

CONCLUSION

Applicants believe that this application is now in condition for allowance. If there are any fees due in connection with the filing of this paper, please charge the fees to Deposit Account No. 08-3425. If a fee is required for an extension of time under 37 C.F.R. § 1.136, such an extension is requested and the fee should also be charged to Deposit Account No. 08-3425.

Respectfully submitted,

Dated: 17 January 2003

Lin J. Hymel (Reg. No. 45,414)
Attorney for Applicants

Human Genome Sciences, Inc.
9410 Key West Avenue
Rockville, MD 20850
Telephone: (301) 251-6015
Facsimile: (301) 309-8439

KKH/LJH/rmr

IL-1 Signaling Cascade in Liver Cells and the Involvement of a Soluble Form of the IL-1 Receptor Accessory Protein[1,2]

Liselotte E. Jensen,[3]*† Marta Muzio,† Alberto Mantovani,† and Alexander S. Whitehead*

The proinflammatory cytokine IL-1 induces the biosynthesis of a number of immunologically important proteins during infection, tissue damage, and/or stress, in part through the activation of the transcription factor NF-κB. Signal transduction is initiated at the cell membrane by complex formation between extracellular IL-1 and the transmembrane IL-1R type I (IL-1RI) and IL-1R accessory protein (IL-1RAcP). The intracellular signaling cascade involves recruitment of two IL-1R-associated kinases, IRAK1 and IRAK2, and the adapter protein MyD88, events which are dependent on the intracellular domain of membrane-bound IL-1RAcP (mIL-1RAcP). In mouse liver, IL-1RAcP is expressed as a soluble protein (sIL-1RAcP), the function of which is unknown. We have cloned the human sIL-1RAcP and established by sequence analysis that the human sIL-1RAcP mRNA arises from alternative splicing of the IL-1RAcP gene (shown here to encompass 12 exons spanning more than 56 kb). Furthermore, we demonstrate that human HepG2 hepatoma cells express both mIL-1RAcP and sIL-1RAcP and that signal transduction in these cells is mediated through IRAK1, IRAK2, and MyD88. We show that phorbol esters induce a change in the pre-mRNA splice pattern such that sIL-1RAcP mRNA becomes the dominant form. Overexpression of a membrane-anchored fusion protein of sIL-1RAcP and MHC in HepG2 cells inhibits IL-1-mediated NF-κB activation, whereas coexpression of IL-1RI with membrane-anchored sIL-1RAcP restores the capacity of the cells to respond to IL-1. This suggests that sIL-1RAcP may act as an inhibitor of IL-1 by directly interacting with IL-1RI to abolish its capacity to transduce signal. *The Journal of Immunology,* **2000, 164: 5277–5286.**

Interleukin-1 is a pleiotropic cytokine that induces multiple physiological responses after infection, tissue damage, and/or stress (reviewed in Ref. 1). A central consequence of cell stimulation by IL-1 is the activation of the transcription factor NF-κB, which mediates the increased transcription of numerous genes and hence increased synthesis of a range of proteins, many of which are involved in immunological responses, e.g., the κ light chain of Ig (from which the transcription factor is named (2)) and the major acute phase reactant serum amyloid A protein (reviewed in Ref. 3).

There are two receptors for IL-1. The type I IL-1R (IL-1RI)[4] has an extracellular portion of 319 amino acid residues comprising three Ig domains, a transmembrane region of 20 amino acid residues, and an intracellular domain of 213 residues (4), whereas the IL-1R type II (IL-1RII) resembles the type I receptor in that it has three extracellular Ig domains (330 amino acid residues) and a transmembrane region of 26 residues, but differs significantly by having only 29 intracellular amino acid residues (5). Both receptors associate with the IL-1R accessory protein (IL-1RAcP) (6–8). IL-1-mediated signaling occurs only via the type I receptor. In contrast, the type II receptor, due to its truncated intracellular domain, acts as a ligand sink (9–11).

The best-described signaling cascade initiated by IL-1 involves a trimeric protein complex of IL-1, IL-1RI, and IL-1RAcP (reviewed in Ref. 12). This complex recruits the adapter protein MyD88 via the intracellular domains of IL-1RI and IL-1RAcP, possibly through protein interactions involving regions of sequence similarity to the C terminus of MyD88 (13). Two kinases, IL-1R-associated kinase (IRAK) 1 and 2, are subsequently recruited, and the signaling cascade further progresses through the TNFR-associated factor 6 (TRAF6), the mitogen-activated protein kinase kinase kinase TGF-β-activated kinase 1, and the NF-κB-inducing kinase (NIK). NIK, and possibly TGF-β-activated kinase 1, is part of the inhibitor of NF-κB (IκB) kinase complex that additionally contains the two IκB kinases IKK-α and IKK-β as well as NF-κB in association with IκB; these may be held together by the scaffold proteins IKK-γ and IKK complex-associated protein (13–21). After the IκB in the IKK complex is phosphorylated, it becomes degraded by the ubiquitin-proteasome pathway, and NF-κB can then translocate to the nucleus where it activates transcription by binding to specific binding sites in the promoters of numerous genes (see Ref. 12 for references). Several kinases are activated by IL-1, and the involvement of a small G protein in IL-1 signaling has been suggested. However, the mechanism whereby these components interact with the above cascade has not been determined (for review, see Ref. 12). Phosphatidylinositol 3-kinase appears to associate with the cytoplasmic region of IL-1RAcP and to initiate a parallel signaling pathway leading to phosphorylation of NF-κB subunits, an event essential for full activition of the transcription factor (22).

*Department of Pharmacology and Center for Pharmacogenetics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; and †Mario Negri Institute for Pharmacological Research, Milan, Italy

Received for publication October 5, 1999. Accepted for publication March 1, 2000.

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[1] This work was supported in part by a European Molecular Biology Organization short-term fellowship (to L.E.J.) and was funded in part by European Community contract no. BIO4-CT97-2107 (New Molecular Targets in the IL-1 System).

[2] The sequences presented in this article have been submitted to GenBank under the accession numbers AF167333–AF167343.

[3] Address correspondence and reprint requests to Dr. Liselotte E. Jensen, Department of Pharmacology, University of Pennsylvania School of Medicine, 156 Johnson Pavilion, 3620 Hamilton Walk, Philadelphia, PA 19104-6084. E-mail address: jensen@pharm.med.upenn.edu

[4] Abbreviations used in this paper: IL-1RI, IL-1R type I; IL-1RII, IL-1R type II; IL-1RAcP, IL-1R accessory protein; IRAK, IL-1R-associated kinase; TRAF6, TNFR-associated factor 6; NIK, NF-κB-inducing kinase; IκB, inhibitor of NF-κB; IKK, IκB kinase; sIL-1RAcP, soluble IL-1RAcP; mIL-1RAcP, membrane-bound IL-1RAcP; RSV, Rous sarcoma virus; PDD, phorbol 12,13-didecanoate; UTR, untranslated region.

0022-1767/00/$02.00

The IL-1RAcP is structurally very similar to IL-1RI and IL-1RII and shares ~25% sequence identity with each (6). Its extracellular domain of 340 amino acid residues is divided into three Ig domains and the transmembrane and intracellular domains are 29 and 181 amino acid residues, respectively. IL-1RAcP was identified in 1995 as a component that is needed for the IL-1R to be fully functional (6). Many studies have since confirmed that the IL-1RAcP is indeed essential for IL-1-mediated responses such as activation of IRAK, NF-κB, Jun N-terminal kinase, and acid sphingomyelinase and for receptor/IL-1 complex internalization (23–26). However, the exact mechanism whereby IL-1RAcP contributes to the function of the IL-1 binding complex has remained obscure. There have been conflicting reports of the effect of IL-1RAcP on the binding kinetics and dissociation constants of IL-1α and IL-1β (6, 25, 27, 28). The C terminus of MyD88 has sequence similarity to the intracellular domain of IL-1RAcP and somewhat weaker similarity to that of IL-1RI. When MyD88 is overexpressed, it can be coimmunoprecipitated with IL-1RAcP, but not with IL-1RI (13). Additionally, IRAK1 interacts with IL-1RAcP, whereas IRAK2 preferentially coimmunoprecipitates with IL-1RI; the latter may interact indirectly with the IL-1RAcP via a death domain (protein-protein interaction domain described initially for proteins involved in apoptosis) that is present in the N termini of both IRAK2 and MyD88 (13, 29, 30). The reports documenting the above suggest that the intracellular domain of IL-1RAcP is essential for signaling from the IL-1 binding complex, and indeed a deletion mutant of IL-1RAcP lacking the majority of the intracellular domain fails to recruit IRAK1 to the IL-1 binding complex and activate NF-κB (29).

In mice, the hepatic form of IL-1RAcP mRNA is only ~1.8 kb compared with ~5.3 kb in all other tissues analyzed, a size difference that has been attributed to alternative splicing (6). The mouse hepatic IL-1RAcP lacks the membrane and intracellular domains of the membrane-bound form and is presumably secreted. This soluble IL-1RAcP (sIL-1RAcP) has so far only been described in mouse liver, and its function has not been determined (6). However, it seems unlikely that sIL-1RAcP would be able to participate in signal transduction from the IL-1 binding complex because it lacks the intracellular domain. This raises the question of how signal transduction takes place in hepatocytes. To address this we have cloned the human homologue of the hepatic sIL-1RAcP and investigated its relationship to the IL-1RAcP gene structure, its function in IL-1-mediated NF-κB activation, and its expression during stress responses.

Materials and Methods

Cloning of the sIL-1RAcP

A human acute-phase liver cDNA library (31) was screened using the oligonucleotide 5′-AGTGATCCTCTGAATGCC-3′ (from human membrane-bound IL-1RAcP (mIL-1RAcP); GenBank accession no. AB006537, position 477–460 in Fig. 1*A*), and a partial cDNA clone encoding the C-terminal 218 amino acid residues and the 767-bp 3′-untranslated region (UTR) of sIL-1RAcP was identified. The remaining sequence corresponding to the 138 N-terminal amino acids of sIL-1RAcP was obtained by RT-PCR using a forward primer (5′-CAAAGGATGACACTTCTGTGG-3′) comprising six nucleotides of the Kozak sequence and 15 nucleotides of the coding region derived from GenBank accession no. AB006537 and a reverse primer (5′-GACCGCATCTATTACCTTTC-3′) specific for the soluble form (position 1066–1047 in Fig. 1*A*). Genomic DNA from HepG2 cells, isolated by conventional methods, and plasmid DNA containing mIL-1RAcP cDNA were used as negative controls to validate the specific amplification of sIL-1RAcP. The PCR product encoding the N-terminal region of sIL-1RAcP was ligated to the sIL-1RAcP-specific C-terminal region at a unique internal Sun I restriction site.

Sequencing of the IL-1RAcP gene

The choice of primers dispersed throughout the cDNA was based on sequence in GenBank accession no. AB006537. Gene fragments were amplified using Expand Long Template, Expand High Fidelity PCR systems (Boehringer Mannheim, Indianapolis, IN) or Human GenomeWalker Kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's instructions. Human genomic DNA (Boehringer Mannheim) or DNA from HepG2 cells were used as templates for long-template PCR. PCR products were gel purified (Qiagen, Santa Clarita, CA) and sequenced. All new sequences reported here and deposited in the GenBank database were sequenced in both directions. Sequence was obtained for the entire available cDNA.

DNA constructs

The Rous sarcoma virus (RSV) promoter was amplified by PCR (32) and cloned into the Renilla-luciferase pRL-null vector (Promega, Madison, WI). The deletion mutants IRAK1(1–217) of IRAK1 and TRAF6(289–522) of TRAF6 were generated by RT-PCR from total RNA isolated from epithelial KB cells and were cloned into the expression vector pCI-neo (Promega). Primers for IRAK1(1–217) 5′-GCGGCAGCCATGGCCGG GG-3′ (forward) and 5′-TCACTTGAGCTCCTCCGAGAAGTTGTGG-3′ (reverse) were derived from GenBank accession no. L76191. Primers for TRAF6(289–522) 5′-ATCTCAGAGGTCCGGAATTTCC-3′ (forward) and 5′-CTATACCCTGCATCAGTAC-3′ (reverse) were derived from GenBank accession no. U78798. A membrane-bound deletion mutant of mIL-1RAcP (IL-1RAcP(1–403)) was generated from an existing construct containing mIL-1RAcP cDNA by PCR using the primer pair 5′-TCAGAA CGCTGCGATGACTGG-3′ (forward) and 5′-TCACAGTAATACAAAT TCTTCTTCTTCC-3′ (reverse) and was cloned into the pFlag-CMV-1 expression vector (Kodak, New Haven, CT). The coding regions for pro-sIL-1RAcP and mature sIL-1RAcP were subcloned into pCI-neo and pFlag-CMV-1, respectively. The fusion protein sIL-1RAcP-MHC was generated by RT-PCR amplification of the conserved transmembrane and intracellular domains (74 C-terminal amino acid residues and stop codon) of MHC class II (GenBank accession no. AF016641) with the forward primer 5′-GGGCTGCCGAAGCCCCTC-3′ and the reverse primer 5′-TCAAGCT GTGAGAGACACATCAG-3′ and by insertion of the product in frame after the coding region of sIL-1RAcP. A control plasmid was generated by cloning the above MHC product into empty pFlag-CMV-1. The nucleotide sequences of cloned constructs were verified by sequencing. The NF-κB-luciferase reporter construct containing the E-selectin promoter ((−730) − (+52)) and the expression constructs specifying Flag-Ag-tagged IL-1RI, Flag-IL-1RAcP, MyD88(152–296), IRAK2(97–590), and NIK(KK429–430AA) are described elsewhere (13).

Cell lines and transfections

Human hepatoma cells (HepG2) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM with 25 mM HEPES and glutamax-1 (L-alanyl-L-glutamine) supplemented with 10% (v/v) FCS, 1 mM sodium pyruvate, 0.01 mM nonessential amino acids, and 50 μg/ml gentamicin (Life Technologies, Grand Island, NY). Cells were grown to ~50% confluence and transfected with 2–3 μg total DNA using the calcium phosphate precipitation method. In experiments in which cells were transfected with varying amounts of expression vectors, the total DNA used in each transfection was held constant by cotransfecting appropriate amounts of empty vector. Cells were allowed to recover in fresh medium for 24 h, after which they were treated with medium (control), IL-1β (10 ng/ml; National Cancer Institute, Frederick, MD), or TNF-α (50 ng/ml; Zeneca Pharmaceuticals, Macclesfield, U.K.) for 6 h. Cells were lysed and lysates were assayed for luciferase and Renilla luciferase activity according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega). Before RNA extraction, cells were treated with IL-1β, TNF-α, and the phorbol esters PMA and phorbol 12,13-didecanoate (PDD) (Sigma, St. Louis, MO).

Northern blotting and quantitative RT-PCR

Isolation of total RNA, Northern blotting, and RT-PCR were performed as described elsewhere (33). Reverse transcription of 1 μg total RNA was performed at 50°C using avian myeloblastosis virus reverse transcriptase and oligo$(dT)_{15}$ primer (Promega) and ANTI-RNase RNase inhibitor (Ambion, Austin, Tx). Quantitative PCR was performed as described elsewhere (33). A 357-bp product was amplified from sIL-1RAcP mRNA with the forward primer QF (5′-GATGGATTCTCGCAATGAGG-3′) and the reverse primer QRS2 (5′-ACTATGGGTTAGATGCCGTC-3′). A 305-bp

A

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   1  ATGACACTTC TGTGGTGTGT AGTGAGTCTC TACTTTTATG GAATCCTGCA
  51  AAGTGATGCC TCAGAACGCT GCGATGACTG GGGACTAGAC ACCATGAGGC
 101  AAATCCAAGT GTTTGAAGAT GAGCCAGCTC GCATCAAGTG CCCACTCTTT
 151  GAACACTTCT TGAAATTCAA CTACAGCACA GCCCATTCAG CTGGCCTTAC
 201  TCTGATCTGG TATTGGACTA GGCAGGACCG GGACCTTGAG GAGCCAATTA
 251  ACTTCCGCCT CCCCGAGAAC CGCATTAGTA AGGAGAAAGA TGTGCTGTGG
 301  TTCCGGCCCA CTCTCCTCAA TGACACTGGC AACTATACCT GCATGTTAAG
 351  GAACACTACA TATTGCAGCA AAGTTGCATT TCCCTTGGAA GTTGTTCAAA
 401  AAGACAGCTG TTTCAATTCC CCCATGAAAC TCCCAGTGCA TAAACTGTAT
 451  ATAGAATATG GCATTCAGAG GATCACTTGT CCAAATGTAG ATGGATATTT
 501  TCCTTCCAGT GTCAAACCGA CTATCACTTG GTATATGGGC TGTTATAAAA
 551  TACAGAATTT TAATAATGTA ATACCCGAAG GTATGAACTT GAGTTTCCTC
 601  ATTGCCTTAA TTTCAAATAA TGGAAATTAC ACATGTGTTG TTACATATCC
 651  AGAAAATGGA CGTACGTTTC ATCTCACCAG GACTCTGACT GTAAAGGTAG
 701  TAGGCTCTCC AAAAAATGCA GTGCCCCCTG TGATCCATTC ACCTAATGAT
 751  CATGTGGTCT ATGAGAAAGA ACCAGGAGAG GAGCTACTCA TTCCCTGTAC
 801  GGTCTATTTT AGTTTTCTGA TGGATTCTCG CAATGAGGTT TGGTGGACCA
 851  TTGATGGAAA AAAACCTGAT GACATCACTA TTGATGTCAC CATTAACGAA
 901  AGTATAAGTC ATAGTAGAAC AGAAGATGAA ACAAGAACTC AGATTTTGAG
 951  CATCAAGAAA GTTACCTCTG AGGATCTCAA GCGCAGCTAT GTCTGTCATG
1001  CTAGAAGTGC CAAAGGCGAA GTTGCCAAAG CAGCCAAGGT GAAGCAGAAA
1051  GGTAATAGAT GCGGTCAGTG ATGAATCTCT CAGCTCCAAA TTAACATTGT
1101  GGTGAATAAG GACAAAAGGA GAGATTGAGA ACAAGAGAGC TCCAGCACCT
1151  AGCCTGACGG CATCTAACCC ATAGTAATGA ATCAAACTTA AATGAAAAAT
1201  ATGAAAGTTT TCATCTATGT AAGATACTCA AAATATTGTT TCTGATATTG
1251  TTAGTACCGT AATGCCCAAA TGTAGCTAAA AAAATCAACG TGAGTACAGT
1301  GAGACACAAT TTTGTGTCTG TACAATTATG AAAAATTAAA AACAAAGAAA
1351  ATATTCAAAG CTACCAAAGA TAGAAAAAAC TGGTAGAGCC ACATATTGTT
1401  GGTGAATTAT TAAGACCCTT TTAAAAATCA TTCATGGTAG AGTTTAAGAG
1451  TCATAAAAAA GATTGCATCA TCTGACCTAA GACTTTCGGA ATTTTTCCTG
1501  AACAAATAAC AGAAAGGGAA TTATATACCT TTTAATATTA TTAGAAGCAT
1551  TATCTGTAGT TGTAAAACAT TATTAATAGC AGCCATCCAA TTGTATGCAA
1601  CTAATTAAGG TATTGAATGT TTATTTTCCA AAAATGCATA ATTATAATAT
1651  TATTTTAAAC ACTATGTATC AATATTTAAG CAGGTTTATA ATATACCAGC
1701  AGCCACAATT GCTAAAATGA AAATCATTTA AATTATGATT TTAAATGGTA
1751  TACACATGAT TTCTATGTTG ATAGTACTAT ATTATTCTAC AATAAATGGA
1801  AATTATAAAG CCTTCTTGTC AGAAGTGCTG CTCCTAAAAA AAAAAAAAAA
1851  AAAAAAA
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B

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Mouse sIL-1RAcP   1 MGLLWYLMSLSFYGILQSHASERCDDWGLDTMRQIQVFEDEPARIKCPLF 50
Human sIL-1RAcP   1 MTLLWCVVSLYFYGILQSDASERCDDWGLDTMRQIQVFEDEPARIKCPLF 50

Mouse sIL-1RAcP  51 EHFLKYNYSTAHSSGLTLIWYWTRQDRDLEEPINFRLPENRISKEKDVLW 100
Human sIL-1RAcP  51 EHFLKFNYSTAHSAGLTLIWYWTRQDRDLEEPINFRLPENRISKEKDVLW 100

Mouse sIL-1RAcP 101 FRPTLLNDTGNYTCMLRNTTYCSKVAFPLEVVQKDSCFNSAMRFPVHKMY 150
Human sIL-1RAcP 101 FRPTLLNDTGNYTCMLRNTTYCSKVAFPLEVVQKDSCFNSPMKLPVHKLY 150

Mouse sIL-1RAcP 151 IEHGIHKITCPNVDGYFPSSVKPSVTWYKGCTEIVDFHNVLPEGMNLSFF 200
Human sIL-1RAcP 151 IEYGIQRITCPNVDGYFPSSVKPTITWYMGCYKIQNFNNVIPEGMNLSFL 200

Mouse sIL-1RAcP 201 IPLVSNNGNYTCVVTYPENGRLFHLTRTVTVKVVGSPKDALPPQIYSPND 250
Human sIL-1RAcP 201 IALISNNGNYTCVVTYPENGRTFHLTRTLTVKVVGSPKNAVPPVIHSPND 250

Mouse sIL-1RAcP 251 RVVYEKEPGEELVIPCKVYFSFIMDSHNEVWWTIDGKKPDDVTVDITINE 300
Human sIL-1RAcP 251 HVVYEKEPGEELLIPCTVYFSFLMDSRNEVWWTIDGKKPDDITIDVTINE 300

Mouse sIL-1RAcP 301 SVSYSSTEDETRTQILSIKKVTPEDLRRNYVCHARNTKGEAEQAAKVK-- 348
Human sIL-1RAcP 301 SISHSRTEDETRTQILSIKKVTSEDLKRSYVCHARSAKGEVAKAAKVK-- 348

Mouse sIL-1RAcP 349 --GNGCTEPMTL*
Human sIL-1RAcP 349 QKGNRCGQ**
Human mIL-1RAcP 349 QKVPAPRYTVELACGFGATVLLVVILIVVYHVYWLEMVLFY 389
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FIGURE 1. Sequence of human sIL-1RAcP and alignment with mouse sIL-1RAcP and human mIL-1RAcP. *A*, Nucleotide sequence of human sIL-1RAcP cDNA. The alternative splice site is underlined at position 1050–1053. The polyadenylation site is dotted underlined at position 1791–1796 and the poly(A) tail is double underlined at position 1836–1857. *B*, The polypeptide sequence of human sIL-1RAcP is aligned with mouse sIL-1RAcP. Vertical lines indicate sequence identity, whereas single or double dots indicate low or high similarity, respectively. The last two and the first 39 aa (including the putative transmembrane domain) after the alternative splice site of human mIL-1RAcP are shown below the sequence of the sIL-1RAcP.

product was amplified from mIL-1RAcP with the QF primer and the reverse primer QRM (5′-TGAGAATCACCACTAGCAGG-3′). PCR products were analyzed on SYBR Green I-stained (Molecular Probes, Eugene, Oregon) agarose gels using STORM and ImageQuant technology (Molecular Dynamics, Sunnyvale, CA).

Results

Cloning of sIL-1RAcP

A human acute-phase liver cDNA library (31) was screened with the oligonucleotide 5′-AGTGATCCTCTGAATGCC-3′, which corresponds to antisense sequence in the extracellular domain of the published sequence of mIL-1RAcP. A clone (L1), which shares sequence identity with human mIL-1RAcP (414–1051 in Fig. 1*A*) at its 5′ end but which has a unique 3′ end of 806 nucleotides, was identified (from 1052 in Fig. 1*A*). The 3′ end contains a polyadenylation site at position 1791–1796 (Fig. 1*A*) and also includes 22 residues of the poly(A)-tail, suggesting that this clone represents a uniquely expressed mRNA. A potential alternative splice site is present at position 1050–1054, where the sequence of L1 diverges from that of mIL-1RAcP; this marks the region where the sIL-1RAcP diverges from the membrane-bound form.

A RT-PCR strategy was applied to obtain cDNA sequence corresponding to the N terminus of sIL-1RAcP. A forward primer was designed for the six nucleotides upstream of the translation start site and the first 15 nucleotides of the coding region of the mIL-1RAcP. A reverse primer was designed across the potential alternative splice site at position 1066–1047 in Fig. 1*A*. A single RT-PCR product of ~1 kb was obtained from HepG2 total RNA. To

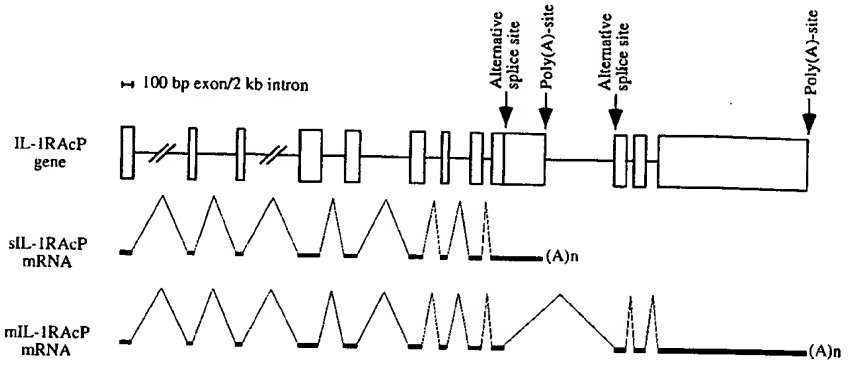

FIGURE 2. Structure of the IL-1RAcP gene. Positions of intron-exon boundaries were identified from sequencing of PCR products obtained from long-range PCR amplification from human genomic DNA using primers distributed throughout the cDNA sequence of mIL-1RAcP (GenBank accession no. AB006537). Exons are shown as boxes, introns as thin lines, and mRNAs as thick lines. (A)n represents the poly(A) tails. The scale bar indicates 100 bp for exon sequence and 2 kb for intronic sequence.

verify that this product could only be derived from sIL-1RAcP mRNA, control amplification reactions using either HepG2 genomic DNA or a plasmid containing the mIL-1RAcP cDNA were included. These latter PCR reactions did not give any products (not shown). The specific PCR product was fused with the clone isolated from the cDNA library at an internal Sun I restriction site and was cloned into the expression vector pCI-Neo.

The first 350 amino acid residues of the soluble and membrane-bound forms of human IL-1RAcP are identical. Clone L1, isolated from the cDNA library, revealed that sIL-1RAcP has an additional six residues C-terminal to the common region, giving a full-length polypeptide of 356 including the signal peptide and a mature protein of 336 amino acid residues. In comparison to this, mIL-1RAcP has an additional 10 extracellular, 29 transmembrane, and 181 intracellular amino acid residues (6). The C-terminal six amino acid residues of the human sIL-1RAcP are four residues shorter than the corresponding region of mouse sIL-1RAcP. Overall, the full-length human sIL-1RAcP polypeptide sequence has 85% identity and 90% similarity to the mouse homologue (Fig. 1*B*).

Structure of the IL-1RAcP gene and alternative splice sites

It has been suggested that the soluble form of IL-1RAcP could arise from alternative splicing (6). To investigate this possibility we designed a forward PCR primer specific for the 3′ end of the common region of the two forms of IL-1RAcP and two forward primers specific for the 3′ ends of the 3′ UTR of each. Reverse primers specific for each of the membrane and soluble 5′ ends were designed.

Using human genomic DNA as template, an ~17-kb PCR product (gAcP25) was amplified using the sIL-1RAcP 3′ UTR forward primer in combination with the mIL-1RAcP 5′ reverse primer. Sequencing of gAcP25 confirmed the presence of additional 5′ mIL-1RAcP-specific sequence and revealed the presence of a 1301-bp intron that divides this sequence into two exons (Fig. 2). Intronic sequence (~15 kb) was found upstream of the mIL-1RAcP- and downstream of the sIL-1RAcP-specific sequences. An ~2.5-kb PCR product (gAcP4) was obtained using the common forward primer with a reverse primer specific for the sIL-1RAcP 3′ UTR; this yielded the entire sIL-1RAcP-specific sequence contiguous with the common region (Fig. 2). An intron of 1.9 kb was identified in the common region amplified in gAcP4.

Primers distributed throughout the full-length mIL-1RAcP cDNA sequence (GenBank accession no. AB006537; a complete list of primers may be obtained from the authors upon request) were used to defined the remaining exon/intron boundaries. In two regions (5′ UTR and signal peptide-mature protein encoding regions), no products could be amplified using conventional PCR with two gene-specific primers. In these regions, a genome walker strategy from Clontech Laboratories was applied. Sequences covering the entire cDNA sequence were obtained from the resulting PCR products, and 12 exons spanning over 56 kb were identified. The 5′ UTR is specified by exons 1 and 2 (Fig. 2 and Table I), and the signal peptide is encoded by exon 3. The first Ig domain is encoded by a single exon (exon 4; Fig. 2 and Table I). Ig domains 2 and 3 are each encoded by two exons (exons 5 and 6 and exons 8 and 9, respectively; Fig. 2 and Table I) and are separated by a small additional exon (exon 7; Fig. 2 and Table I) of 72 bp. The exon encoding the second half of Ig domain 3 is followed by the exon encoding the C-terminal region and 3′ UTR of sIL-1RAcP. An intron of ~15 kb separates the sIL-1RAcP 3′ UTR from the exon encoding the transmembrane domain (exon 10; Fig. 2 and Table I) of mIL-1RAcP. The intracellular domain of mIL-1RAcP is encoded by two exons (exons 11 and 12; Fig. 2 and Table I), of which the second also encodes the 3′ UTR of mIL-1RAcP. All introns follow the GT-AG rule. One sequence discrepancy, an insertion of TA, relative to the cDNA sequence (AB006537) was found at position 2336. The boundaries of exon 5 are in agreement with those previously submitted to GenBank (accession no. AF016261) in connection with mapping of the IL-1RAcP gene to chromosome 3 (34).

Engagement of signaling cascade components downstream of the IL-1 binding complex

To determine which signaling pathway is utilized to transduce signal from the IL-1R and activate NF-κB in human HepG2 cells, we overexpressed dominant-negative mutants of early components in the IL-1 signaling pathway and measured the activity of NF-κB after treatment with IL-1β or TNF-α using a NF-κB-luciferase reporter construct. TNF-α was used as a control to ensure that effects were specific to the IL-1 signaling pathway. The TNF and IL-1 signaling pathways leading to NF-κB activation are initially distinct but converge at NIK, i.e., NIK is activated by both IL-1 and TNF, whereas the earlier components analyzed in this study should be specific to the IL-1 signaling cascade. Cells transfected with empty vector together with the NF-κB-luciferase reporter and RSV-Renilla construct exhibited a strong NF-κB activity as measured by the NF-κB reporter construct when treated with IL-1β compared with cells treated with medium only (15- to 150-fold induction). Treatment of similarly transfected cells with TNF-α resulted in NF-κB activities of ~50% of those achieved by IL-1β treatment. The significance of results was assessed using standard *t* test.

Cotransfection of cells with a construct encoding a kinase-inactive NIK, NIK(KK429–430AA), together with the NF-κB reporter and RSV-Renilla constructs resulted in changed IL-1β and TNF-α responses (Fig. 3*A*) that were both dependent on the concentration of the NIK inactive construct. At the lowest concentrations (0.01 μg/transfection) an increase in IL-1β-induced NF-κB activity was

Table I. *Structure of the IL-1RAcP gene*[a]

Exon	Segment/ Domain	mRNA Position (bp)	Exon Length (bp)	5′ Intron-Exon Junction	3′ Exon-Intron Junction	Intron Length (kb)
1	5′UTR	1–118	118	TGCCGGGATCCAGGTCTCCG	CTCTCAGCTTCCCAAGAAAG	ND
2	5′UTR	119–205	87	ndGCATCGTCATGTGATCATCA	gtgagtctcgcgccgccgtg CTCCCTTTAATATCTCAAAG	8
3	Signal peptide	206–270	65	ctttgtatttatggttacag	gtaattcattttctgattta CCTGCAAAGTGATGCCTCAG	ND
4	Ig 1	271–556	286	GATGACACTTCTGTGGTGTG ttctttttgattattcca	gtaagtgaatggcttttgac AACTATACCTGCATGTTAAG	4.5
5	Ig 2A	557–743	187	gAACGCTGCGATGACTGGGGA ttgtatatttctttttcag	gtagcctgattcttggcagt CGACTATCACTTGGTATATG	10
6	Ig 2B	744–909	166	GAACACTACATATTGCAGCA ttttttttactttttctag	gtaaggaaaattagactaca TCTGACTGTAAAGGTAGTAG	3
7		910–981	72	GGCTGTTATAAAATACAG ttttatttcctctaacacag	gtaagcatgattagtgtcca GGTCTATGAGAAAGAACCAG	4
8	Ig 3A	982–1108	127	GCTCTCCAAAAAATGCAGTG ttttgggtttttttttcag	gtaattacagctatgtctct GATGTCACCATTAACGAAAG	1.9
9	Ig 3B	1109–1257	149	GAGAGGAGCTACTCATTCCC tttcttctctctatttctag	gtatcatgggggccagcaag AGCCAAGGTGAAGCAGAAAG[AS]	0/15
9	Soluble C-terminal and 3′UTR	1258–2041	784	TATAAGTCATAGTAGAACAG [AS]GTAATAGATGCGGTCAGTGA	[pAsig]AATAAATGGAAATTATAAAGCCT TCTTGTCAGAAGTGCTGCTCCT[pAsite]	
10	Transmembrane	1258–1407	150	ttctttgttgttcattccag[AS]	aaatcagactgtgtctaaaa AACAGATGAAACCATTTTAG	1.3
11	Intracellular	1408–1551	144	TGCCAGCTCCAAGATACACA atctgtgttccttttgctag	gtaagtaacagaaatttgac AGACAGTCTGCCTGGGGGAA	2.5
12	Intracellular and 3′UTR	1552–4726	3175	ATGGAAAAGAGTATGATATT atggttgtttttctttcccag	gtaggtatttcagaggagta [pAsig]AATAAACCATTTATTTTCAGCTTTG[pAsite]	
				TTGTCACAGATGAGACTTTG		

[a] Positions of intron-exon boundaries were identified by sequencing the products of long-range PCR amplification from human genomic DNA using primers distributed throughout the cDNA sequence of mIL-1RAcP (GenBank accession no. AB006537). Position numbers are as in the mIL-1RAcP cDNA, except for the exon for the soluble IL-1RAcP C-terminal and 3′UTR. AS, Alternative splice site; Ig, immunoglobulin domain; nd, intronic sequence not determined; ND, intron size not determined; pAsig, polyadenylation signal; pAsite, polyadenylation site.

detected in two of three independent experiments. The significance of this observation is unknown. At the highest concentration (1.25 μg/transfection), the IL-1β- and TNF-α-induced NF-κB activities were reduced to ~35% ($p < 0.01$) and 30% ($p < 0.001$), respectively, relative to that in control cells transfected with empty vector.

A dramatic but graded reduction of the IL-1β response from ~60% ($p < 0.001$) of the full response in control cells (Fig. 3*B*) to ~25% ($p < 0.001$) at the lowest and highest concentrations of cotransfected DNA, respectively, was achieved by overexpression of a deletion mutant of TRAF6, TRAF6(289–522), which lacks a RING finger and five potential zinc-finger motifs that are believed to be involved in protein-protein interactions with other members (most probably NIK) of the signaling cascade (14). The TNF-α-induced response remained virtually unchanged (Fig. 3*B*). This suggests that both NIK and TRAF6 are involved in the IL-1 signaling cascade in HepG2 cells.

Components recruited to the IL-1 binding complex

To investigate whether the IL-1 binding complex functionally requires MyD88 and the IRAK1 and IRAK2 kinases as previously reported, we overexpressed dominant-negative deletion mutants of these proteins. A deletion mutant of MyD88, MyD88(152–296), which lacks the death domain, reduced the IL-1β-induced NF-κB response to ~25% ($p < 0.001$; at highest concentrations of 1.25 μg/transfection) of that in control cells (Fig. 4*A*) in a specific and concentration-dependent manner. In contrast, the TNF-α-induced response remained constant. This indicates that MyD88 is involved in IL-1β-mediated signaling leading to NF-κB activation but not in that mediated by TNF-α.

A reduction in readout was achieved by cotransfecting as little as 2 ng/transfection (Fig. 4*B*) of a deletion mutant of IRAK1 lacking the kinase domain (IRAK1(1–217)). NF-κB activity could be further reduced using higher amounts of this construct, up to 250 ng/transfection, at which the IL-1β-induced response was only ~20% ($p < 0.001$) of that in control cells. No inhibition of the TNF-α-induced response is apparent (Fig. 4*B*), suggesting that, as with MyD88, IRAK1 is only involved in the IL-1β-mediated NF-κB activation and not in that mediated by TNF-α. A deletion mutant of IRAK2 (IRAK2(97–590)) lacking the death domain could also specifically, but to a lesser extent (reduction to ~50%; $p < 0.001$), inhibit the IL-1β response (Fig. 4*C*).

Taken together, these results suggest that the "signaling complex" recruited to the IL-1 binding complex is assembled as previously described (13, 15).

Components in the IL-1 binding complex

We further analyzed the effects of overexpressing components in the IL-1 binding complex itself. The cells used in these studies were treated with IL-1β or TNF-α at concentrations that would produce an optimal response (10 ng/ml and 50 ng/ml, respectively) or were treated with a lower concentration of IL-1β that was only sufficient to produce a response 10 times lower than that of the optimal IL-1β concentration.

After introducing as little as 0.1 ng/transfection of an IL-1RI expression construct, we observed an ~2-fold ($p < 0.01$) increase in the cellular response to the suboptimal concentration of IL-1β (Fig. 5*A*), and using 2 ng/transfection the cellular response could be further augmented such that the "suboptimal" IL-1β concentration could produce the same effect as the "optimal" IL-1β concentration. Overexpression of IL-1RI had no measurable effect on cells treated with optimal amounts of either IL-1β or TNF-α (Fig. 5*A*). In addition, overexpression of mIL-1RAcP (up to 1.25 μg/transfection) had no effect on IL-1β or TNF-α-treated cells (not

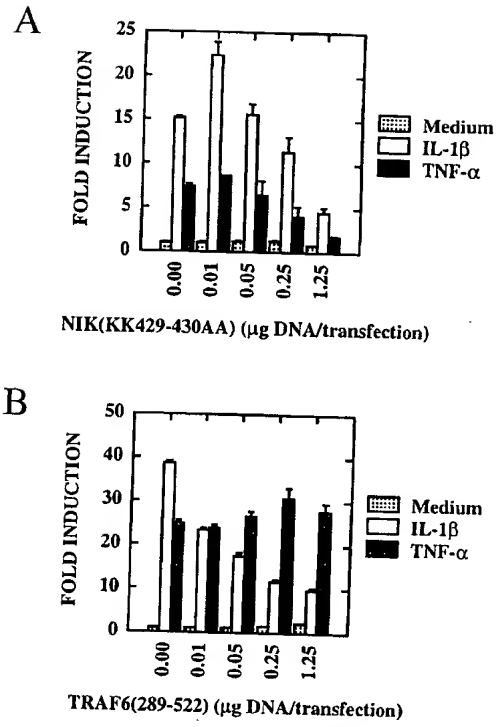

FIGURE 3. IL-1β signaling is mediated through NIK and TRAF6. HepG2 cells were cotransfected with 0.5 μg NF-κB-luciferase reporter plasmid, 3 ng RSV-Renilla plasmid, and the indicated concentrations of expression constructs. Luciferase activity was determined 6 h after treatment with medium (control), IL-1β, or TNF-α and was standardized against Renilla-luciferase activity. Relative luciferase activity is represented as fold induction compared with cells transfected with NF-κB-luciferase reporter plasmid, RSV-Renilla plasmid, and empty vector and treated with medium only. Transfections were performed in triplicate and the results shown are representative of at least three independent experiments. *A*, A dominant-negative NIK(KK429–430AA) inhibits activation of NF-κB mediated by IL-1β (open bars) and TNF-α (grey bars) in a concentration-dependent manner. Medium only controls are shown as dotted bars. *B*, A dominant-negative TRAF6(289–522) specifically inhibits activation of NF-κB mediated by IL-1β (open bars) but not by TNF-α (grey bars) in a concentration-dependent manner.

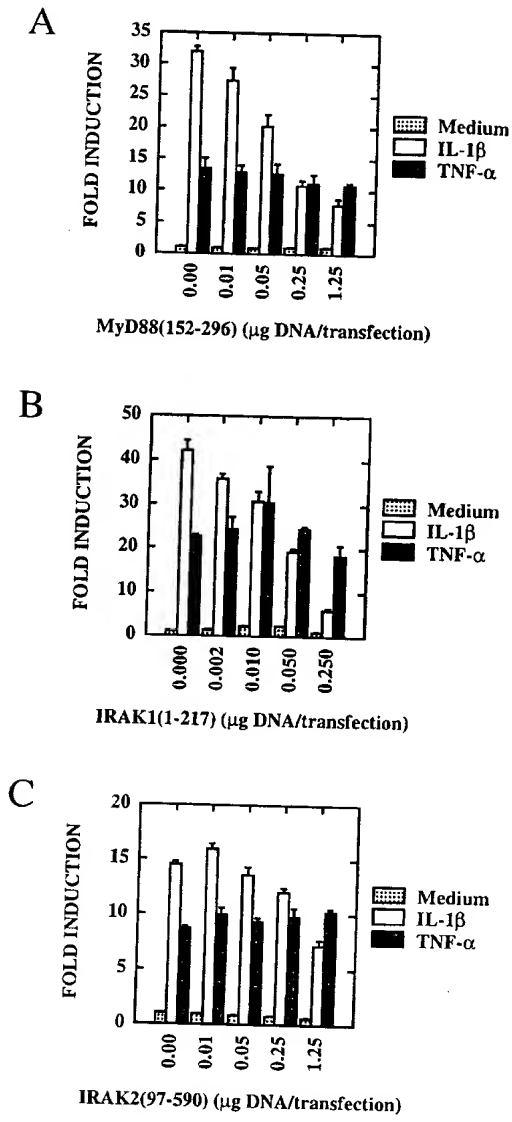

FIGURE 4. Dominant-negative mutants of components recruited to the IL-1 binding complex inhibit IL-1β signaling. HepG2 cells were cotransfected as described in Fig. 3. Relative luciferase activity is represented as fold induction compared with cells transfected with NF-κB-luciferase reporter plasmid, RSV-Renilla plasmid, and empty vector and treated with medium only. Transfections were performed in triplicate and the results shown are representative of at least three independent experiments. Dominant-negative mutants MyD88(152–296) (*A*), IRAK1(1–217) (*B*), and IRAK2(97–590) (*C*) specifically inhibit activation of NF-κB mediated by IL-1β (open bars) but not by TNF-α (grey bars) in a concentration-dependent manner. Medium only controls are shown as dotted bars.

shown). This suggests that it is the number of IL-1RI molecules, and not IL-1RAcP, on the cell membrane that determines the magnitude of the cellular response to IL-1.

Initial experiments using cells transfected with sIL-1RAcP expression constructs did not show any effects that could be attributed to secreted sIL-1RAcP. We speculated that this could be due to dilution of the soluble protein in the tissue culture medium to a low concentration at which its biological activity would be below detectable limits. Therefore, to increase its local concentration at the cell surface we fused the sIL-1RAcP to the transmembrane and intracellular portion of MHC class I, which is conserved in MHC I molecules. When a construct encoding sIL-1RAcP-MHC was cotransfected into cells, a significant concentration-dependent decrease in the response to IL-1β was observed (Fig. 5*B*). At the highest amounts of transfected sIL-1RAcP-MHC used (1.25 μg/transfection), the responses to suboptimal and optimal amounts of IL-1β were reduced to ~5% ($p < 0.001$) and 10% ($p < 0.01$), respectively, relative to controls. No significant change in the response to TNF-α was observed (Fig. 5*B*). Cotransfection of a deletion mutant of mIL-1RAcP (IL-1RAcP(1–403)) lacking the entire intracellular domain gave similar results (not shown). Furthermore, cotransfection of a control plasmid, CMV-Flag-MHC, expressing only the MHC domain of the sIL-1RAcP-MHC fusion protein had no such effect (not shown).

When cells are cotransfected with both IL-1RI and mIL-1RAcP, a spontaneous activation of NF-κB can be observed in cells treated with medium only ($p < 0.001$; Fig. 5*C*). This spontaneous activation has been used in some previous studies of the IL-1 signaling cascade (13) as the model system of IL-1R complex activation. However, such spontaneous activation does not take place when cells are cotransfected with IL-1RI and sIL-1RAcP-MHC (not shown). When cells are cotransfected with a fixed amount of construct encoding sIL-1RAcP-MHC and increasing amounts of IL-1RI construct, a pattern somewhat similar to the one observed

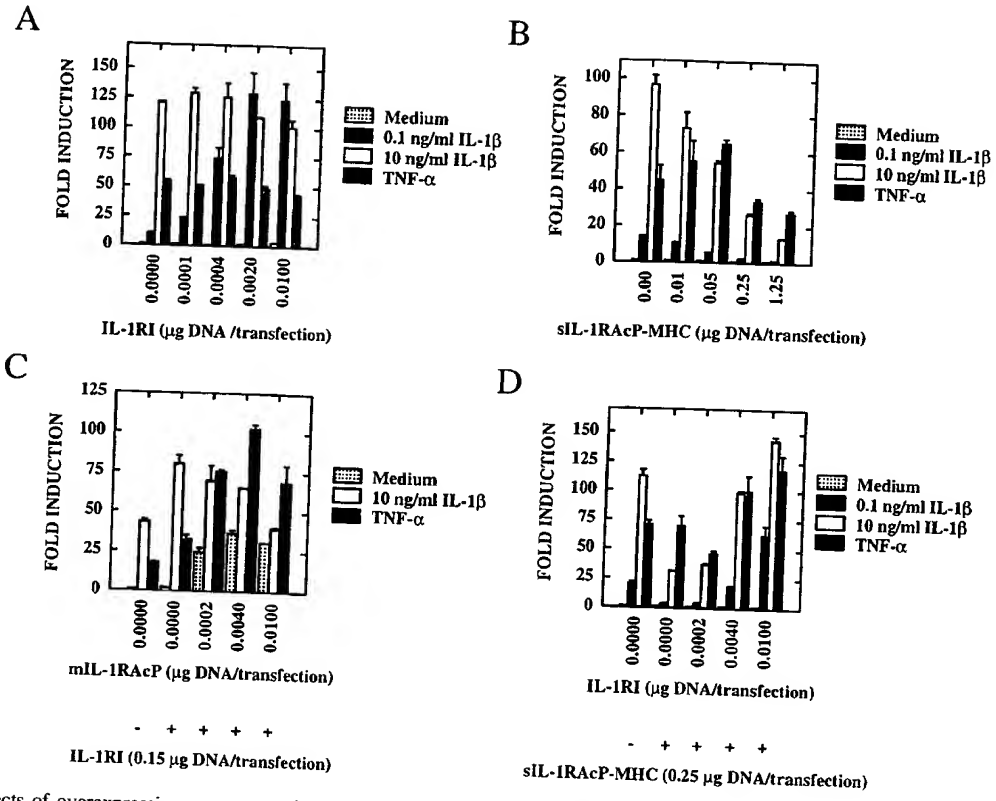

FIGURE 5. Effects of overexpressing components in the IL-1 binding complex. HepG2 cells were cotransfected and relative luciferase activity was represented as described in Fig. 3. Transfected cells were treated with medium (control, dotted bars, not visible), 0.1 ng/ml IL-1β (filled bars), 10 ng/ml IL-1β (open bars), or 50 ng/ml TNF-α (grey bars). *A*, Cells transfected with a full-length and functional IL-1RI showed an increased sensitivity to IL-1β with the suboptimal concentration of 0.1 ng/ml IL-1β after transfection with increasing amounts of IL-1RI construct (filled bars). *B*, The fusion protein sIL-1RAcP-MHC inhibits the IL-1β-induced NF-κB activation in a concentration-dependent manner. *C*, Introduction of mIL-1RAcP into cells transfected with 150 ng IL-1RI (bars labeled with "+") causes concentration-dependent spontaneous activation of NF-κB in cells treated with medium only (dotted bars). *D*, Introduction of IL-1RI into cells transfected with 250 ng sIL-1RAcP-MHC (bars labeled with "+") reestablishes the sensitivity of the cells to IL-1β in a concentration-dependent manner.

when cells are transfected with the IL-1RI construct alone is seen. In cells transfected with 250 ng of the sIL-1RAcP expression construct, transfection of 2 ng of IL-1RI expression construct is sufficient to bring NF-κB activation after treatment with both suboptimal and optimal amounts of IL-1β back to the level of that observed in control cells transfected with the empty vector (Fig. 5*D*). However, it is not sufficient to cause the shift of "suboptimal-to-optimal" level of activation seen when the cells are transfected with the IL-1RI construct alone. Transfection of 10 ng IL-1RI construct into cells cotransfected with the sIL-1RAcP-MHC construct results in an ~2-fold ($p < 0.01$) increase in response to the suboptimal amount of IL-1β compared with control cells transfected with empty vector (Fig. 5*D*). This is 100 times more than is needed to cause the same change in the IL-1β response of cells transfected with the IL-1RI construct only. Similar results were obtained when increasing amounts of the mIL-1RAcP construct were cotransfected with a fixed amount of sIL-1RAcP-MHC (not shown).

These results suggest that it is the number of available IL-1RI molecules that determines cellular sensitivity to IL-1β and that sIL-1RAcP may act as an inhibitor of IL-1β by rendering the IL-1RI nonfunctional in a signaling context, although it is probably still able to bind IL-1β.

Expression of membrane and soluble IL-1RAcP mRNA in human hepatoma cells and acute-phase liver

Experiments using conventional Northern blot analysis to determine which form of the IL-1RAcP is expressed in hepatoma cells were not sensitive enough to detect any mRNA. Therefore, we developed a quantitative semicompetitive RT-PCR in which sIL-1RAcP and mIL-1RAcP cDNA products can be amplified using the same forward primer in combination with reverse primers specific for each form; this approach generates 357-bp and 305-bp PCR products, respectively. The first 233 bp of the two PCR products are identical and the relative amounts may be assessed by amplifying both sIL-1RAcP and mIL-1RAcP in the same reaction. To confirm that an accurate determination of the ratio of the two splice variants can be established using this method, plasmids encoding sIL-1RAcP and mIL-1RAcP were mixed in different ratios. Aliquots were removed after different numbers of amplification cycles and were analyzed on SYBR Green-stained 2.5% agarose gels. The ratios of sIL-1RAcP product to mIL-1RAcP product remained unchanged during both the exponential and plateau phases of amplification (not shown).

We then analyzed RNA samples extracted from HepG2 hepatoma cells after treatment with the phorbol esters PDD and PMA for 1–96 h using the above quantitative PCR method. In cells treated with medium only (0 h; Fig. 6), mIL-1RAcP mRNA constituted approximately two-thirds of the total amount of IL-1RAcP cDNA. PDD elicited a gradual change in the ratio of the two alternative splice variants over time: after 12 h of incubation with PDD, mIL-1RAcP and sIL-1RAcP mRNAs were present in equal amounts, whereas after 24 and 36 h, the sIL-1RAcP splice form had become the dominant species (Fig. 6). After 72 h, at which time the cells still appeared healthy, the ratio showed a reversal in

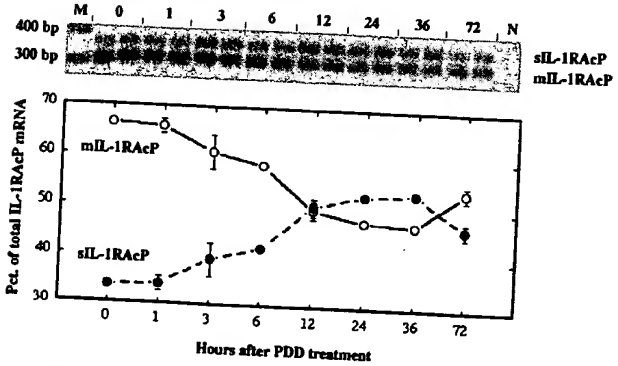

FIGURE 6. Expression of mIL-1RAcP and sIL-1RAcP mRNA in response to phorbol esters. After reverse transcription with oligo(dT), the relative abundance of mIL-1RAcP and sIL-1RAcP cDNAs was determined using a semicompetitive quantitative approach in which mIL-1RAcP and sIL-1RAcP cDNAs were coamplified using common forward and isoform-specific reverse primers. The PCR products generated from sIL-1RAcP and mIL-1RAcP cDNAs are 357 bp and 305 bp, respectively. HepG2 cells were treated with 50 ng/ml of the phorbol ester PDD, and RNA was harvested after various time points between 1 and 72 h. *Top panel*, SYBR Green-stained agarose gel of duplicate RNA samples for each time point. Lane marked "M" represents size markers of 300 and 400 bp, respectively. Lane marked "N" represents negative control for reverse transcription and PCR. Time points are indicated above the respective duplicate lanes. Graph shows the relative abundance of the two IL-1RAcP mRNA species as determined using STORM and ImageQuant technology (Molecular Dynamics). sIL-1RAcP and mIL-1RAcP are shown with filled symbols and dashed line and open symbols and solid line, respectively.

trend toward normal. The change induced by PDD was concentration dependent (not shown). PMA gave rise to a similar, but less dramatic, change in the ratio between the two mRNA species (not shown).

Discussion

mIL-1RAcP is an accessory protein to the IL-1R that has recently been identified (6). It has been established that mIL-1RAcP is necessary for IL-1-induced signaling from the IL-1RI leading to activation of NF-κB, but the mechanism whereby it contributes to this process is still largely unknown. In addition to the membrane-bound form of IL-1RAcP, a soluble form of the protein has also been identified (6). The sIL-1RAcP has so far only been identified in mouse liver (6), mouse T cell lymphomas (25), and rat brain (35), and its function has remained obscure. We have cloned the human sIL-1RAcP and demonstrated that it arises from alternative splicing. The relative proportion of alternatively spliced forms of IL-1RAcP mRNA in HepG2 cells changes in response to stress and/or acute-phase induction and becomes biased toward sIL-1RAcP mRNA. We hypothesize that sIL-1RAcP acts as an inhibitor of IL-1 signal transduction by associating with the IL-1RI to reduce or eliminate its signaling capacity.

Contradictory hypotheses have been offered as to whether it is the level of membrane expression of IL-1RI or of IL-1RAcP that regulates the cellular response to IL-1, but no definitive data to resolve this issue have been reported (8, 36). HepG2 cells express high levels of IL-1RII and low levels of IL-1RI (Ref. 37 and L. E. Jensen, unpublished observation). This leads to strong competition between the two receptors for binding IL-1 when this cytokine is present at suboptimal levels (the suboptimal concentration of 0.1 ng/ml used in this paper is similar to the maximum physiological concentrations of 0.2–0.5 ng/ml in human blood during sepsis (1)). Increasing the cell surface expression of IL-1RI would shift the balance toward more IL-1/IL-1RI complexes and would result in a higher level of NF-κB activation, which is in accordance with our observations. Because the increased expression of mIL-1RAcP reported here had no influence on the efficiency of signal transduction, it is likely that the level of IL-1RI is the variable that controls the response to IL-1. Our observations that expression of a membrane-anchored form of sIL-1RAcP results in almost 100% inhibition of the NF-κB activation that can be induced by IL-1 at physiological concentrations and that this inhibition can be reversed/prevented by coexpression of IL-1RI suggest that IL-1RI is regulated at the membrane by sIL-1RAcP such that, although all of the IL-1RI molecules are capable of binding IL-1, only a limited number are actually able to mediate signal transduction. This interaction constitutes an additional layer of receptor competition. Given that we have only been able to achieve an effect on the level of NF-κB activation with the membrane-anchored form of sIL-1RAcP, we cannot exclude the possibility that the native protein may have an additional function. A component downstream of the IL-1 binding complex might be an additional limiting factor because NF-κB activation is clearly saturated at optimal concentrations of IL-1. However, we do not believe that this is physiologically significant because the optimal concentration of IL-1 in cell culture experiments is probably very much higher than that achieved in vivo.

It is not known precisely how IL-1RAcP interacts with the IL-1RI. We have demonstrated that overexpression of a membrane-anchored form of the sIL-1RAcP in which both the transmembrane and small intracellular domains are unrelated to the equivalent domains in mIL-1RAcP results in specific inhibition of the IL-1 signaling cascade. We have also observed that a deletion mutant of IL-1RAcP that lacks the entire intracellular domain can also inhibit IL-1-mediated signaling. This establishes that extracellular interactions between IL-1RAcP and IL-1RI are sufficient to mediate the association of the two proteins, leading to measurable biological effect. The possibility that there are additional interactions between the transmembrane and intracellular domains is not formally excluded; however, such interactions, if they do occur, are not necessary for the biological effects reported here.

There are currently two models for the interaction among IL-1RI, IL-1RAcP, and IL-1. In the first model, IL-1RI and IL-1RAcP are already associated and the subsequent binding of IL-1 results in a conformational change in IL-1RI that initiates the downstream signaling cascade. In the second model, IL-1RI and IL-1RAcP become associated only after IL-1 binds to IL-1RI and may involve a permissive conformational change that facilitates recruitment of IL-1RAcP or down-stream signaling components. However, the observation that overexpression of IL-1RI and mIL-1RAcP together causes a "spontaneous activation" of the IL-1 signaling cascade (reported in this paper and elsewhere (13)) suggests that such an IL-1-mediated conformational change is not essential for activation of the signaling cascade and provides good evidence in favor of the second model. Under this model, IL-1RI and mIL-1RAcP will only rarely come together by chance because they are normally expressed at low levels and spontaneous activation, therefore, does not occur; however, when both proteins are overexpressed, random association and spontaneous activation becomes more likely. In nontransfected cells with normal levels of membrane components, IL-1 may promote an increased affinity between IL-1RI and mIL-1RAcP to produce a complex that is sufficiently stable to allow the recruitment of additional intracellular signaling components.

Only a limited number of studies of IL-1RAcP expression has been published. Bacterial LPS causes a moderate up-regulation of mIL-1RAcP mRNA in human monocytes (38), and induction of

mIL-1RAcP mRNA has been demonstrated in lung, spleen, and thymus tissue from mice injected with IL-1 (6). High levels of constitutive mIL-1RAcP expression have been demonstrated in mouse brain tissue (6); however, another group has reported that in rats, IL-1 causes an up-regulation in the expression of both sIL-1RAcP and mIL-1RAcP in brain tissue (see Ref. 35 and its references). In this latter study, no significant change in the ratio of mIL-1RAcP to sIL-1RAcP mRNA was reported. Expression of mouse liver sIL-1RAcP mRNA has previously been shown to be constitutive (6). Our results establish that both mIL-1RAcP and sIL-1RAcP mRNAs, generated by alternative splicing, are expressed in human HepG2 cells; however, in untreated cells, the ratio of mIL-1RAcP mRNA to sIL-1RAcP mRNA is ~2:1. The ratio of these alternatively spliced mRNAs changes significantly after treatment with inflammatory mediators. The phorbol esters PMA and PDD, wide spectrum inducers of nitric oxide synthase, and protein kinase C activation (39, 40) induce an early response (3–36 h) in which the ratio becomes biased toward sIL-1RAcP mRNA. Factors that control tissue and developmentally specific alternative splicing have previously been identified (see Ref. 41 for references), and such a factor may direct the alternative splicing of IL-1RAcP pre-mRNA in liver cells; if so, the activity of the factor could be controlled by extracellular stimuli. Another mechanism whereby the ratio of the two IL-1RAcP mRNA species may be altered is differential stability. The 3′ UTRs of the sIL-1RAcP and mIL-1RAcP mRNAs, respectively, contain two and 14 AUUUA sequence motifs (L. E. Jensen, unpublished observation) that are common in mRNAs of components of the inflammatory response and have previously been shown to confer instability (42, 43). Therefore, the mIL-1RAcP mRNA may be significantly less stable than sIL-1RAcP mRNA during the acute-phase response; rapid degradation of mIL-1RAcP mRNA would yield an IL-1RAcP mRNA profile that is dominated by sIL-1RAcP mRNA.

IL-1 is a proinflammatory cytokine involved in the early stages of inflammation and the acute-phase response (1). Extracellular signals received by liver cells during the acute-phase response, analogous to those we generated by the phorbol esters PMA and PDD, may change the bias in alternative splicing of the IL-1RAcP pre-mRNA to permit the sIL-1RAcP mRNA to become predominant, thereby limiting the cellular response to IL-1.

The IL-1RI and IL-1RII genes are believed to be derived from a common ancestor because the positions of introns relative to sequence encoding the extracellular regions are almost identical (44). The IL-1RAcP gene structure resembles that of IL-1RI in that 1) two exons specify the 5′ UTR, 2) the signal peptide is encoded by one exon, and 3) the extracellular, transmembrane, and intracellular regions are encoded by six, one, and two exons, respectively. In addition, the second intracellular exon also encodes the 3′ UTR. Introns are positioned at similar positions within the two genes. The genomic arrangement of the IL-1RII gene is consequently very similar but distinguishes itself by not having an intron between the 5′ UTR and signal peptide-encoding regions (44). The IL-1RI gene distinguishes itself by having a shorter exon 4 encoding the N terminus of the mature protein than the equivalent exons 4 and 3, respectively, in the IL-1RAcP and IL-1RII genes do. Unlike the IL-1RI and IL-1RII genes, which are both located on chromosome (45), the IL-1RAcP gene maps to chromosome 3 (34). All of the above suggest that the IL-1RI, IL-1RII, and IL-1RAcP genes have evolved from a common ancestor and have adopted features required to mediate different functions.

From the IL-1RAcP gene structure we have been able to determine that the two forms of IL-1RAcP arise from alternative splicing and that the sIL-1RAcP mRNA is derived from the first nine exons, whereas mIL-1RAcP mRNA arises from splicing of exon 10 to exon 9 at an internal splice site in exon 9 (Table I). This internal splice site is seven nucleotides further downstream of the one suggested by Greenfeder et al. (Ref. 6 and Fig. 1*B*). A splice site similar to the one identified by us in humans is also present in the mouse mIL-1RAcP cDNA sequence. Unfortunately, the mouse sIL-1RAcP nucleotide sequence and complete amino acid sequence have never been published, and it is therefore not possible to determine whether different splice sites for the mIL-1RAcP are used in mice and humans.

In this paper we have reported the cloning of the human sIL-1RAcP and have characterized the IL-1RAcP gene that encodes the two alternatively spliced forms (sIL-1RAcP and mIL-1RAcP). In addition, we have shown that a membrane-anchored form of sIL-1RAcP inhibits IL-1-mediated cell activation and that the splicing pattern of the IL-1RAcP pre-mRNA changes in response to stress. This suggests that IL-1RAcP is a very important factor in controlling the IL-1 response. Future work on the biological activity of the native sIL-1RAcP and characterization of the tissue-specific expression of mIL-1RAcP and sIL-1RAcP and the mechanism whereby the above switch is triggered during the acute-phase response will enhance our understanding of IL-1-mediated signaling and may contribute to our overall understanding of the biochemical basis of cytokine and hormone actions in general.

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